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Can Lignin Be Accurately Measured?

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ABSTRACT

Forages serve an important role in providing nutrients to ruminants while providing positive benefits to the environment. Forage cell wall digestibility is incomplete because of several structural features within the wall, but digestion is mostly inversely correlated with the amount of lignification that has occurred during cell wall development. Lignin is a hydrophobic polymer formed through enzyme-mediated radical coupling of monolignols, mainly coniferyl and sinapyl alcohols. The polymer is highly resistant to degradation and generally passes through the ruminant unmodified. Though lignin is resistant to degradation, it is not easily quantified within various types of forages. Numerous methods have been developed over the years to measure lignin levels in different plant species. Most frequently used among workers involved with forage development or utilization are the acid detergent, Klason, and permanganate lignin methods. More recently, acetyl bromide has received attention as a possible lignin determination method. The acetyl bromide method is dependent on determining the absorbance of the extract in which all the lignin of a sample has been dissolved. Each of these methods gives different lignin values for the same type of forage sample. For example, acid detergent, Klason, permanganate, and acetyl bromide lignin methods give quite different values for alfalfa stems: 93, 145, 158, and 135 g lignin kg^{-1} cell wall, respectively. These differences can be even greater for grasses: 25, 77, 45, and 92 g kg^{-1} cell wall from corn (*Zea mays* L.) stalks analyzed by acid detergent, Klason, permanganate, and acetyl bromide lignin methods, respectively. This paper will discuss the different lignin determination methods and highlight the advantages and disadvantages of each as they relate to forage sample analysis.

Lignification is the biochemical process of forming the collective of phenylpropanoid macromolecules termed lignin (Sarkanen and Ludwig, 1971). Lignin can be defined from a chemical point of view (i.e., its chemical composition and structure) or from a functional view that stresses what lignin does within the plant. It has been recognized for over 50 yr that lignin is a polymeric material composed of phenylpropanoid units derived primarily from three cinnamyl alcohols (monolignols): *p*-coumaryl, coniferyl, and sinapyl alcohols (Sarkanen and Ludwig, 1971). With the development of molecular techniques, it has been possible to manipulate the lignification process by altering enzymes within the monolignol biosynthesis pathway resulting in the incorporation of nontraditional residues into lignin polymers (Sederoff et al., 1999). The general chemical definition might be too simplistic (Sarkanen and Ludwig, 1971), and there are now many examples showing that other phenolics besides the cinnamyl alcohols can be incorporated into lignins (as reviewed by Sederoff et al., 1999). From a functional point of view, lignins impart strength to cell walls, facilitate water transport, and impede the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects, and other herbivores.

It is important, no matter which definition you prefer, to be able to determine the concentration of lignin within a wide range of cell wall types. Since lignin is fairly resistant to both chemical and biological degradation, one would think that it would be relatively easy to measure (Sarkanen and Ludwig, 1971). Lignin has been defined, at least in general chemical terms, for 50 yr and several well-defined procedures to quantify lignin in plant tissues have been developed and approved as AOAC (Association of Analytical Communities International formerly Association of Official Analytical Chemists) or standard wood chemistry methods. Of the several types of methods available to determine the lignin in plant samples, not one would be considered a standard unambiguous method for all samples. With the increased interest in altering lignin quantity and composition for a variety of reasons (increased ease of pulping, digestibility, etc.), plus availability of molecular techniques to accomplish this task, come the following questions. What method should be used to measure lignin? Does changing the lignin composition by adding unique phenolics alter the properties sufficiently to warrant concern with the reliability of a favorite method? We will review the different types of lignin methods that have been used, focusing on the main procedures that are used for herbaceous plants.

Methods for Determining Lignin

There have been numerous methods developed and modified through the years to quantitatively determine the amount of lignin that is in a given type of plant tissue. Methods for determining lignin in samples can be divided into two basic categories, those that rely on a gravimetric determination and those that are based on a noninvasive method. Many procedures that have been developed (modified) through the years have their roots in the wood chemistry and paper and pulp industries. In some cases, they have been adapted, or attempts have been made to adapt them, to be used with herbaceous samples. Some of the methods discussed here require that the plant sample be subjected to some form of extraction to remove potentially interfering compounds. This probably is the one step that has been modified the most in terms of adapting procedures developed for woody species (or wood derived samples) for use with herbaceous samples. As always, there are

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exceptions to this generalization. Attempts through the years have been made to develop noninvasive techniques for the determination of lignin content in plant samples so that there is no risk of chemical alteration of the lignin.

Noninvasive Methods

For these types of analyses, attempts have been made to exploit the chemical properties of lignin to absorb radiation in discrete regions of the electromagnetic spectrum. The production of unique spectra in these regions allows one to select specific spectral characteristics (wavelengths) in which the response will be proportional to the amount of lignin. Early attempts tried to capitalize on the stronger absorbance of lignin components compared with carbohydrates in the ultraviolet (UV) region of the spectrum. Bolker and Somerville (1962) measured lignin content of finely ground wood samples incorporated into potassium chloride pellets. Though sample preparation was relatively easy, consistency of sample incorporation into uniform pellets was a problem. Application to herbaceous plant samples is problematic because of the frequently high amounts of nonlignin phenolics contained in these types of plants. A modification of this technique that has some application is the use of UV-microspectrophotometery to evaluate cell walls of specific plant tissues (Boutelje and Jonsson, 1980; Fergus and Goring, 1970). The amount of lignin within a given cell wall region can be determined by applying Beer's Law, i.e., $A = \varepsilon cd$; where A = absorbance, ε = extinction (absorption) coefficient, c = concentration g L^{-1}, and d = section thickness or length of light path through the tissue section. Determining the appropriate extinction coefficient becomes a problem, particularly when evaluating cell wall types that contain different types of lignin composition, sinapyl:coniferyl: *p*-hydroxycinnamyl alcohols in varying portions. It is not appropriate to assume that the extinction of the free alcohols would accurately represent the extinction coefficient for a polymer that is composed of different ratios of the core alcohols. Fergus and Goring (1970) found that the absorption maxima for guaiacyl- and syringyl-based model compounds were 280 and 270 nm, respectively, adding to the difficulty of using the correct extinction coefficient. Application to forages in digestibility studies has provided insight into the relationship of lignin within a cell wall type and digestibility (Akin and Hartley, 1992). Though it is possible to get a relative concentration of lignin within different cell wall types, it is not possible to extrapolate to a lignin concentration value within the whole tissue of the plant.

Infrared spectroscopy can be a powerful technique for characterizing phenolics, including lignin, in cell wall samples. This technology has been promoted as a possible way of quantifying lignin in samples, particularly with the application of improved techniques such as diffuse-reflectance Fourier transform spectrometry (Schultz et al., 1985). Because energy modes of different bond types (C–O, C=O, O–H, etc. stretching or vibrational energies) are measured, a tremendous amount of information can be obtained from a single sample. When evaluating lignin within the unmodified wall matrix, the most convenient method of analysis, one has to deal with the overlap of peak intensities in certain regions of the spectra. Fortunately, there are spectral regions that are relatively specific to lignin and allow accurate measurement of peak intensities, potentially useful for quantifying lignin. As discussed under UV analysis of lignin, deciding on the appropriate standard for comparison is problematic. With the high sensitivity of the infrared spectroscopy techniques, it is quite easy to compare samples and determine structural and compositional similarities or differences, although relating these observations to total lignin content in the cell wall sample is difficult. This method also has the advantage of requiring only a small amount of sample.

Recently near infrared spectroscopy (NIRS), the range of electromagnetic spectrum that is between the visible and infrared wavelengths, has been utilized as a means of quantifying lignin. This technique is used to estimate the concentrations of various plant components (fats, oils, protein, total fiber, etc.) by correlating spectral changes in this region (generally overtones from IR region) to specific concentrations of the component measured using reference wet chemistry methods. Because of the nature of NIRS statistical analysis, Beer's law does not hold and spectral measurements are not directly related to concentration of lignin. However, the method is sensitive to spectral changes that are related to changes in broader classes of components [protein, neutral detergent fiber (NDF), etc.] within the sample being analyzed. The NIRS method is dependent on the precision of the assays used to identify and quantify the individual components in the original calibration set. This may be one reason that R^2 values for predictive equations of lignin are not as good as some other forage components (Casler and Jung, 1999). The amount of total lignin in the calibration sets (for forages 1–24% depending on the assay used) and the precision of the type of lignin assay impact the predictive value of the NIRS method. Nonetheless it has the advantage of being quite rapid, sample preparation is minimal, and NIRS instruments can be automated for high throughput of samples.

Nuclear magnetic resonance spectroscopy (NMR) is an analytical technique that is frequently used to provide detailed characterization of composition and structural features of lignin, particularly when the lignin can be dissolved into a suitable solvent for solution state NMR. A difficulty in dissolving lignin in solvents is one reason solution state NMR is restricted to analyzing only a portion of the total lignin in a given type of plant sample. Solid state NMR generally lacks the resolution needed for detailed structural analysis because of peak broadening. However, with the development of Cross Polarization/Magic Angle Spinning NMR some of these problems have been overcome and has allowed this technique to be used to analyze lignin in whole samples (Haw et al., 1984). Quantification can be achieved by integration of specific peaks within the total lignin spectrum, but these values must be related back to a standard sample

of known lignin content. New advances have improved solid-state NMR, but it has not been used routinely for quantification of lignin in whole plant samples. This may be due to difficulties in obtaining good clean sharp spectra from complex plant samples and the general high cost for instrumentation that will provide adequate results.

One advantage all of the noninvasive techniques have is that they leave the sample chemically unaltered. This may be a critical aspect if additional information is needed from the sample and quantity is limited. All of the spectral methods discussed above need only a milligram to gram quantities of plant material. Each method above suffers from being dependent on an appropriate "standard lignin" with which to calibrate the instrument. Many times they are dependent on the results from some other assay for the lignin content of a standard lignin. This is a problem that is not unique to these techniques, as we will see later.

Indirect Methods

Over the years, methods have been developed that indirectly indicate the amount of lignin present in a sample, typically wood pulps. These methods take advantage of the chemical nature of lignin and its ability to consume oxidants more readily than other cell wall components (polysaccharides). Under controlled experimental conditions the amount of oxidant consumed during the reaction can be used as a measure of the lignin present in the sample. Typically chlorine or potassium permanganate is used as the oxidant. Originally, a special apparatus was used to measure the amount of gaseous chlorine taken up by a specific weight of pulp sample. It had the advantage of indicating not only the amount of lignin but also the bleaching requirements necessary to produce a high-quality paper (Dence, 1992). This procedure was later modified to use sodium or calcium hypochlorite in an acidic solution and results were based on titration to determine the amount of chlorine consumed (Colombo et al., 1962). As with the chlorine or hypochlorite procedures, the potassium permanganate procedure is based on the addition of an excess of 0.1 *M* $KMnO_4$ in an aqueous solution and measurement of the amount of oxidant consumed at the end of a specific reaction time. Generally, results are recorded as the milliliters of permanganate solution consumed by 1 g of pulp sample. Tasman and Berzins (1957) modified the procedure such that the sample size was adjusted to ensure that approximately half of the added $KMnO_4$ is consumed and correcting the volume of oxidant actually consumed by the sample such that it is exactly 50% of the total applied. This procedure helps correct for differences in lignin content in different samples and the variable amount of oxidant consumed. The result is the "kappa number" and has been adopted as a standard procedure in many pulp and paper organizations (Dence, 1992). Relationships have been developed for the conversion of kappa, permanganate, chlorine, and hypochlorite numbers to Klason lignin values.

Solubilization of Lignin

Two procedures (thioglycolate and acetyl bromide) also rely on the complete solubilization of lignin, but in this case the lignin in solution is measured. We place them in a separate category because of their unique approach to lignin analysis. Both methods depend on sufficient derivatization of lignin to render the lignin soluble in a suitable solvent.

The thioglycolate lignin method involves the formation of thioethers of benzyl alcohol groups typically found in lignin (Fig. 1). Chemically modified lignin formed by the reaction contains acid groups rendering the lignin soluble under alkaline conditions. The original thioglycolate procedure treated 40 g of an extracted cell wall sample with a mixture of 30 g of thioglycolic acid and 400 mL of 2 *M* HCl followed by heating for 7 h at 100°C (Browning, 1967). The resulting suspension was filtered and the insoluble residue washed thoroughly with water before air-drying. After suspending the insoluble residue in ethanol for 48 h, the insoluble residue was filtered and air-dried. Subsequently the residue is stirred with 400 mL of 0.5 *M* NaOH overnight. The

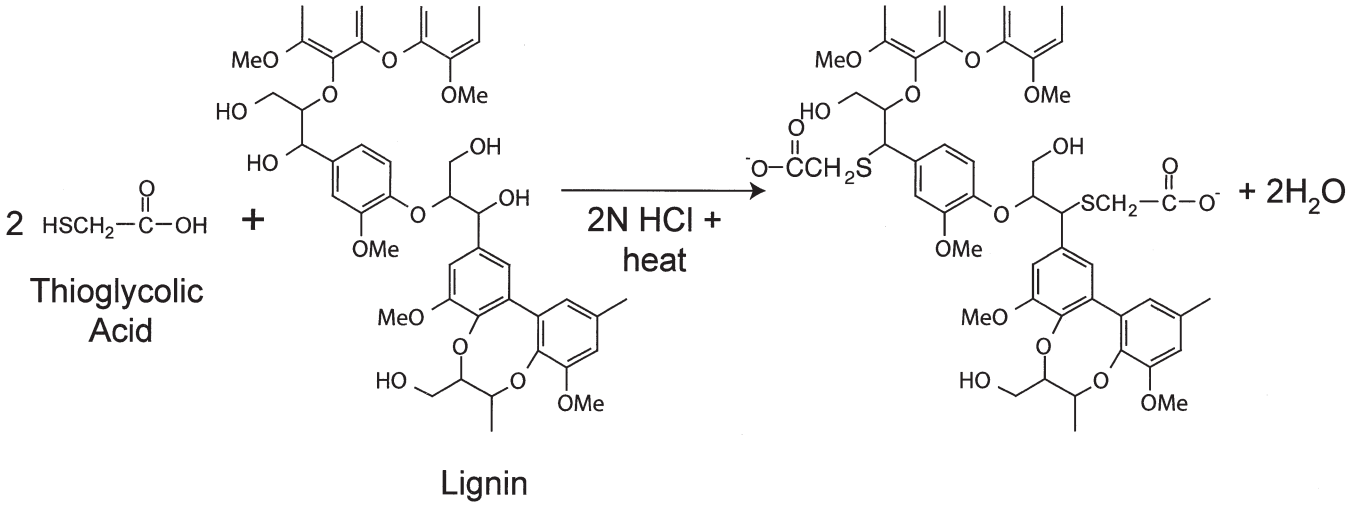

Fig. 1. Reaction mechanism for thio derivatization of lignin by thioglycolate reagent to render lignin soluble under alkaline conditions.

thioglycolate lignin was dissolved in the alkaline solution and separated from the remaining cell wall material by filtration and washing of the residue. The filtrate and washings were acidified with concentrated HCl to precipitate the lignin, and recovered by filtration and washing with water to remove residual acid. This crude lignin preparation was then purified by dissolving in dioxane, filtration, and precipitation from the dioxane solution by dilution with ether. The insoluble lignin is washed with ether and allowed to dry to obtain a recovered weight of thioglycolate lignin. Inclusion of a standard lignin is important to allow correction for mass changes due to the derivatization of the lignin by thio groups.

Recently this procedure has been modified to allow application to samples that are 10 to 15 mg in size (Bonello et al., 1993; Bruce and West, 1989; Lange et al., 1995). Cell wall samples are treated with 1 mL of 2 *M* HCl and 0.2 mL of thioglycolate for 4 h at 95°C. The insoluble residue is recovered by centrifugation, washed three times with water, and the thioglycolate lignin dissolved in 0.5 *M* NaOH. Nonlignin materials are pelleted by centrifugation. Thioglycolate lignin is recovered by acidification with HCl (4 h at 4°C) and centrifugation to pellet the lignin. After drying, the insoluble lignin residue is dissolved in 0.5 *M* NaOH and diluted 40:1 with 0.5 *M* NaOH before reading at 280 nm to ensure the total absorbance remains on scale. Quantification of the lignin is based on absorbance values obtained from a specific quantity of synthetic dehydrogenation lignin polymer (DHP) formed from coniferyl alcohol and radical formation after treatment with horseradish peroxidase. Although originally proposed in 1940 (Browning, 1967), the thioglycolate lignin method has not been widely used perhaps because of the lack of suitable lignin standards required for calibration. A review of the literature shows a renewed interest in this method particularly when applying the adaptations that allow it to be used with milligram samples. One uncertainty of the procedure is the possibility of problems arising from acid soluble lignins. Grasses seem to have significant amounts of acid soluble lignin especially after treatment of cell walls with alkaline solutions (Hatfield, unpublished) that could potentially remain in solution during the acid precipitation steps. If this is the case, one could seriously underestimate the total lignin in such cell wall samples. However, the chemical nature of the thioglycolate derivatives of the lignin may render these lignins insoluble in acid. This would have to be verified with appropriate forage samples.

Johnson et al. (1961) developed a method to dissolve lignin in a solution of acetyl bromide and acetic acid. The method results in the formation of acetyl derivatives of unsubstituted OH groups within a lignin polymer and bromine replacement of α-carbon OH groups rendering the lignin molecule soluble in acetic acid (Fig. 2). The method has been used frequently since its development and modified several times to work with nonwoody plant samples (Bagley et al., 1973; Morrison, 1972a, 1972b). Dence (1992) cited the advantages of the procedure as being rapid and simple, appropriate for small sample size (5–25 mg), with no need to correct for acid soluble lignin, providing precise absorbance values for determining total lignin content, and having less interference from nonlignin products.

The original acetyl bromide method (Johnson et al., 1961) utilized 5 to 100 mg of extracted cell wall material depending on the suspected lignin concentration of the sample. A thoroughly dried sample was placed in a glass reaction vial (15 mL) with 5 mL of 25% (v/v) acetyl bromide in acetic acid, sealed with Teflon lined caps, and heated at 70°C for 30 min. After digestion, the vial's contents were quantitatively transferred to a 100-mL volumetric flask containing 10 mL of 2 *M* NaOH and 25 mL of acetic acid, 1 mL of 7.5 *M* hydroxylamine, and made up to 100 mL with acetic acid. Blanks were run in conjunction with the samples and the UV absorption spectrum measured against the blank. Although the method is based on absorption values at 280 nm, obtaining a full UV spectrum from 250 nm to 400 nm has advantages. If there has been excessive degradation of the sample during the acetylation step, the spectrum

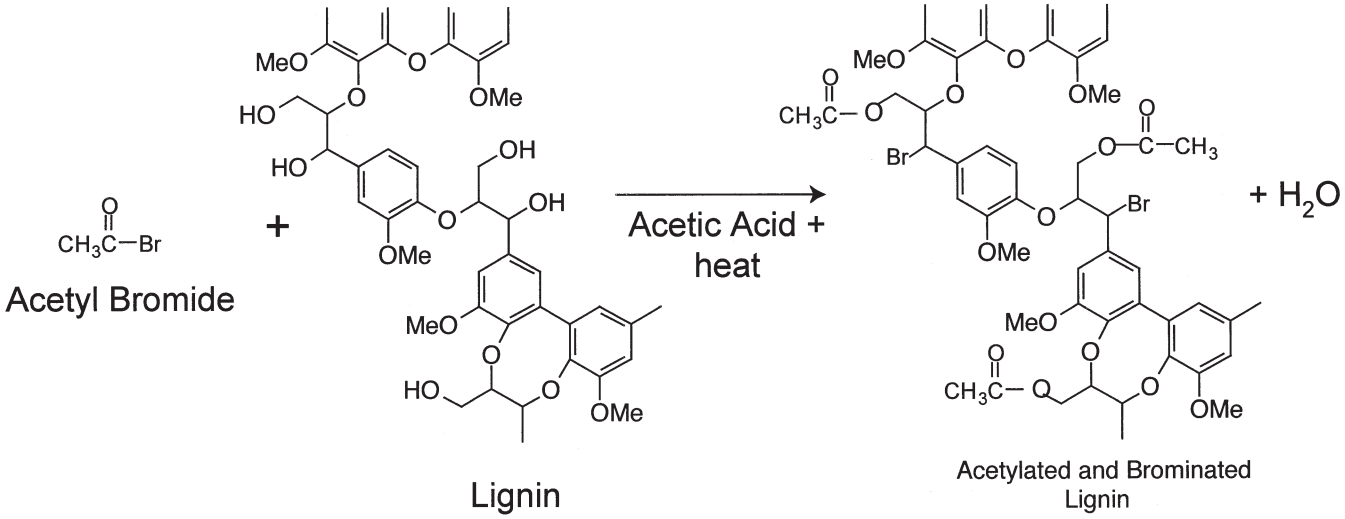

Fig. 2. Reaction mechanism for acetyl derivatization of lignin by acetyl bromide reagent to render lignin soluble under acidic conditions.

Table 1. Lignin coefficients developed for the acetyl bromide lignin method based on acid-dioxane lignins isolated from different plant cell wall material. Data adapted from Fukushima and Hatfield (2004).

Plant material	Growth stage	Mean extinction coefficient
Alfalfa (*Medicago sativa* L.)	full bloom lower 30 cm of stem (FBLS)	14.23
Alfalfa	full bloom upper 30 cm of stem (FBUS)	15.99
Alfalfa	seed development, lower stem (SDLS)	15.30
Alfalfa	prebud stage whole stem (PBWS)	15.69
Red clover (*Trifolium pratense* L.)	full bloom, whole stem	14.49
Bromegrass (*Bromus inermis* Leyss.)	boot stage, whole stem (BSWS)	17.11
Bromegrass	anthesis, whole stem (AnWS)	16.73
Bromegrass	postseed, whole stem (PSWS)	17.40
Cornstalk (*Zea mays* L)	postanthesis, internode 9–12	17.75
Oat straw (*Avena sativa* L.)	leaf (OSL)	20.10
Oat straw	stem (OSS)	18.91
Wheat straw (*Triticum aestivum* L.)	leaf (WSL)	19.81
Wheat straw	stem (WSS	17.54
Overall mean		17.04 ± 1.80

will reflect these changes. In addition, if a lignin sample has high sinapyl alcohol content the absorption maximum will shift toward 270 nm, the maximum for monomeric sinapyl alcohol. Morrison (1972a, 1972b) modified the procedure by introducing filtration steps to remove the haze caused primarily by protein that remains insoluble in the acid solutions. For herbaceous samples that typically have higher protein concentrations than woody plants, even in the extracted cell wall preparations, light scattering and absorption can present problems. Iiyama and Wallis (1988) introduced the use of perchloric acid into the original reaction mixture to help ensure the complete solubilization of cell wall samples. Later Hatfield et al. (1999) cautioned against the use of perchloric acid particularly with herbaceous samples that contained significant amounts of xylans. They found that under the acidic conditions in the presence of acetyl bromide, xylans are partially degraded to furfurals that absorb in the 280-nm region. Perchloric acid greatly exacerbates this degradation, particularly if samples are not completely dry. This seems to be a unique characteristics of xylans, as other polysaccharides did not exhibit the same extent of degradation (Hatfield et al., 1999).

One difficulty with the acetyl bromide method, as with the thioglycolate and other methods already mentioned, is the need for a well defined lignin standard with which one can calibrate the method to obtain the correct absorbance values for quantifying lignin in an unknown sample. Numerous lignin sources have been used as standards for calibrating the procedure with absorptivity at 280 nm ranging form 11.0 to 34.9 L g^{-1} cm^{-1} (Dence, 1992). Fukushima and Hatfield (2001) proposed using lignin extracted with HCl-dioxane as a standard for acetyl bromide calibration. The procedure is easy and straightforward, producing a lignin that is relatively low in contamination by protein and carbohydrates that can be easily quantified and corrected for on a weight basis. Using this procedure, Fukushima and Hatfield (2001) isolated lignins from a range of plant materials and determine the extinction coefficients for each (Table 1).

Direct Methods

Over the years, numerous methods have been proposed using mineral acids to solubilize and hydrolyze carbohydrates in cell wall samples leaving the lignin residue to be determined by gravimetric measurement. By far the most commonly used method for determining lignin is the Klason or 72% (v/v) H_2SO_4 acid procedure. The method was originally developed in the early 1900s by Klason using 64 to 72% H_2SO_4 to dissolve away all of the polysaccharides, leaving lignin as an insoluble residue (Browning, 1967). Briefly, the original procedure for wood involves grinding the dried sample (0.5- to 1-mm screen) and extracting with appropriate solvents to remove the potentially interfering materials that would be insoluble in the acid solution. Carefully weighed (1 ± 0.1 g) dry cell wall samples are suspended in 15 mL of 72% H_2SO_4 stirred with a glass rod while maintaining the temperature at 20°C for 2 h. The total contents are transferred and diluted with distilled water into an Erlenmeyer flask to a final volume of 575 mL. The dilute acid solution is boiled for 4 h under reflux to maintain a constant volume. After cooling, the lignin is collected in a preweighed filtering crucible and washed with water to remove residual acid before drying to a constant weight. Numerous modifications have been proposed over the years, including the use of an autoclave to avoid the need for reflux apparatus (Yoshihara et al., 1984), neutralization of supernatant subsamples after the secondary hydrolysis step for total carbohydrate determinations (Pettersen et al., 1984), neutral sugar composition (Hatfield et al., 1994), and total uronosyls (Hatfield and Weimer, 1995; Hatfield et al., 1994).

For woody samples, adequate extraction can be easily achieved with a range of organic solvents. Herbaceous samples pose a more difficult problem, most notably because of the higher protein contents and the frequent appearance of waxes from cutin when leafy materials are subjected to analysis. Proteins pose the greatest problems particularly in forage plants that may contain 15 to 25% protein in the dry matter (cutins and waxes typically make up only 1% or less of the dry matter). Ellis (1949) proposed for high-protein samples found in immature wood that they be pre-extracted with ethanol-benzene followed by a proteolytic enzyme treatment and diluted sulfuric acid before applying the 72% H_2SO_4 procedure to obtain acid insoluble lignin residue. Goering and Van Soest (1975) proposed using an acid detergent extraction step to produce an acid detergent fiber

(ADF) that is essentially cellulose and lignin with small amounts of pectins and xylans also present depending on the sample and if a neutral detergent extraction had been applied before the AD treatment. This treatment removes many of the potentially contaminating substances from the lignin residue. It does not remove such materials as cutin and suberin. For samples that may contain high levels of these materials Goering and Van Soest (1975) recommended using a permanganate oxidation (similar to the procedure discussed above) to remove the lignin and leave all other materials behind including the cutin and suberin. Since suberins contain phenolic materials along with long chain alcohols the permanganate oxidation could potentially remove part of these materials. Since noncellulosic polysaccharides are more susceptible to permanganate oxidation, residual carbohydrates in the ADF could lead to an overestimation of lignin. A serious drawback of the ADF method is that when applied to grasses the acid detergent solution can effectively dissolve 50% or more of the lignin (Hatfield et al., 1994; Kondo et al., 1987; Lowry et al., 1994). Acid detergent also solubilizes lignin from forage legumes but not to the same extent (Hatfield et al., 1994). Theander and coworkers (Theander, 1983; Theander and Westerlund, 1986) proposed a simplified scheme (Uppsala method) to quickly produce cell wall preparations (alcohol insoluble residues). The procedure involves cycles of sonication in 80% (v/v) ethanol, centrifugation to pellet the insoluble residue, and fresh ethanol extraction. Starches are removed with amylase/amyloglucosidase treatment. If one is interested in analyzing total cell wall polysaccharides, this procedure avoids those that are potentially dissolved away by the detergent system, e.g., pectins. A disadvantage of the system is that a significant portion of the proteins remains with the cell wall fraction and would end up as a possible contamination of Klason lignin determinations on such materials. It might be possible to introduce a protease treatment into the scheme to remove excess protein. Care should be taken when selecting the type of protease so as not to alter the polysaccharide composition of the cell wall residue. Acidic pepsin has been used as a method to remove protein (Ellis, 1949), but this treatment may also remove arabinose units on pectins and arabinoxylans because of the acid labile nature of arabinofuranoysl units (Aspinall, 1982).

Acid Soluble Lignins

In most samples, a small portion of the total lignin may be soluble in the dilute acid solution from the second stage of the Klason lignin procedure. Wood chemists have developed a standard protocol for measuring acid soluble lignin listed as TAPPI Useful Method UM-250 (TAPPI, 1985). Essentially the method relies on determining the UV absorption of the final diluted acid solution of the Klason lignin procedure. Hydrolyzate from the second stage of the Klason lignin procedure is read in a standard UV cuvette (1-cm path length) at 200 to 205 nm. The amount of lignin is determined by Beer's law ($A = \varepsilon cd$), where A = absorbance at 205 or 200nm, ε = absorptivity (L $g^{-1}cm^{-1}$), d = path length (1 cm), and c = concentration (g L^{-1}). There are two problems with this determination. First, the extinction coefficient that is used can vary with the type of lignin and should be determined for each type of lignin that is under study. Since this is not practical, typically a value cited in the literature (110 L $g^{-1}cm^{-1}$, Dence, 1992) may be used to estimate the lignin value. Second, the choice of absorption maximum to use for analysis must be decided. The normal wavelength of 280 nm is not used because of the potential interference from furfural and hydroxymethylfurfural formation from carbohydrates during the acid hydrolysis. These carbohydrate degradation products strongly absorb at 280 nm. Selection of 205 nm or lower wavelengths would pose a potential problem in that carbohydrate monomers begin to absorb light in the 190- to 205-nm range. Even though this absorption is not strong, the abundance of released sugars, particularly in samples with low-lignin contents, may result in an overestimation of this potential lignin fraction. One should approach such determinations of acid soluble lignin with caution.

What Is the Best Method for Determining Lignins?

There does not appear to be a clear winner in terms of providing an accurate measure of the total lignin in a given sample. The use of a particular method may be justified on the basis of laboratory constraints as opposed to one method being clearly better than others. Recently, Fukushima and Hatfield (2004) compared the four most popular methods for measuring lignin in forage samples to determine the lignin content of several different plant samples (Table 2). These data are not presented here as promoting any one method over another; however, these data do provide a comparison of the different lignin methods utilizing the same plant samples. It is clear that the acid detergent lignin (ADL) procedure produced the lowest lignin values of all the methods. It is interesting that much higher values were obtained for the permanganate lignin method that is also based on using acid detergent fiber (ADF). One would have thought that these would have been closer even if the acid detergent had removed some of the lignin before the analysis. Either there is a significant amount of acid soluble lignin, particularly in grasses, or there are other nonlignin components within the wall residues that are oxidized/dissolved by the permanganate solution.

As of now, there is no single method that is rapid, noninvasive, handles large sample numbers, and provides accurate measure of cell wall lignin contents. Probably the most important consideration is consistency; one should use the same lignin method for all of samples being analyzed. This will allow a relative comparison of the samples that should avoid the greatest potential problems that might arise from switching from one method to another. Other procedures such as the Klason method can be adapted to obtain nearly a complete cell wall component analysis from one sample. Some

Table 2. A comparison of mean lignin values (± sd) generated from application of the four different lignin methods to a range of forage samples. All values are given in g kg^{-1} cell wall (Fukushima and Hatfield, 2004). Plant materials are as described in Table 1.

Plant material	Acid detergent	Klason	Permanganate	Acetyl bromide soluble
	g kg^{-1}			
Alfalfa FBLS	92.5 ± 3.0	144.8 ± 0.4	157.6 ± 16.8	134.7 ± 1.6
Alfalfa FBUS	59.3 ± 0.3	111.4 ± 0.6	95.3 ± 10.9	71.4 ± 4.9
Alfalfa SDLS	90.6 ± 1.4	138.8 ± 1.0	153.8 ± 10.5	117.3 ± 2.3
Alfalfa PBWS	83.6 ± 2.0	123.0 ± 3.1	134.7 ± 6.7	116.6 ± 4.3
Red clover	41.7 ± 1.3	71.3 ± 1.5	115.6 ± 8.1	90.4 ± 1.1
Bromegrass BSWS	30.4 ± 0.1	100.4 ± 13.8	64.1 ± 1.7	127.5 ± 0.4
Bromegrass AnWS	36.5 ± 1.3	109.8 ± 15.6	69.9 ± 6.4	144.6 ± 12.9
Bromegrass PSWS	45.6 ± 1.7	130.1 ± 0.1	67.1 ± 1.1	139.1 ± 5.2
Corn stalk	24.8 ± 0.2	76.7 ± 7.8	45.2 ± 13.2	92.0 ± 2.9
Oat straw OSL	106.9 ± 2.8	138.1 ± 1.9	71.3 ± 8.1	123.5 ± 0.3
Oat straw OSS	83.3 ± 0.6	171.2 ± 1.6	111.5 ± 5.4	186.3 ± 5.9
Wheat straw WSL	103.4 ± 10.5	141.6 ± 0.1	74.3 ± 11.6	149.9 ± 3.4
Wheat straw WSS	89.1 ± 10.8	184.2 ± 2.1	122.0 ± 12.9	213.0 ± 15.0

procedures, such as the thioglycolate lignin method, may hold promise of producing a true reflection of lignin, but more research must be undertaken to ensure that all the lignin is precipitated by the acid step.

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